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Phalloidin depletes the mitochondrial Ca^{2+} compartment of hepatocytes

Heinz Faulstich, Klaus Münter and D. Mayer*

Department of Physiology, Max-Planck-Institute for Medical Research, Jahnstr. 29, 6900 Heidelberg and **Division of Cytopathology, Institute of Experimental Pathology, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG*

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In 3 h of incubation, primary cultures of rat hepatocytes attach to the substratum and exchange about 2.2 nmol $^{45}Ca^{2+}$ per mg protein. In the presence of 1 μM phalloidin, the exchanged amount of $^{45}Ca^{2+}$ was found to be decreased by about 30%. Using the uncoupling agent FCCP and the ionophore A23187 for further characterisation we determined that the $^{45}Ca^{2+}$ deficit caused by phalloidin occurs in the FCCP-sensitive compartment, i.e., the mitochondria.

Phalloidin *Mushroom toxin* *Hepatocyte* *Ca^{2+} pool* *Mitochondria*

1. INTRODUCTION

In hepatocytes Ca^{2+} appears to be sequestered mainly in two compartments, the mitochondria and the endoplasmic reticulum, while the Ca^{2+} concentration in cytoplasm is low (0.1–0.3 μM).

Increased cytoplasmic Ca^{2+} concentration has been suggested as the ultimate lesion caused by several hepatotoxic agents [1], among them the mushroom poison phalloidin [2]. In fact, it is tempting to suppose that about 1 mM Ca^{2+} flooding into a cell from the extracellular space down a gradient of about 5000:1, may represent a lethal injury. However, the model has been doubted by others [3], who found that hepatotoxic agents, particularly those consuming glutathione, cause blebbing and cell death by disturbance of the intracellular Ca^{2+} homeostasis [4], rather than penetration of Ca^{2+}. The influx of extracellular Ca^{2+} into hepatocytes was suggested by indirect measurements only. The authors [5] observed an enhanced vulnerability of hepatocytes incubated with 6×10^{-5} M phalloidin in the presence of 3.6 mM Ca^{2+}. This prompted us to determine, by direct measurement, the nature of possible phalloidin-induced Ca^{2+} fluxes in cultured hepatocytes.

Abbreviations: FCS, fetal calf serum; PBS, phosphate-buffered saline; DMPHN, demethylphalloin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

2. MATERIALS AND METHODS

2.1. *Isolation and culturing of hepatocytes*

Hepatocytes were isolated as in [6] with some modifications [7]. In contrast to [6] we used Häms F12, Ca^{2+} and Mg^{2+} free (Biochrom, Berlin), instead of Dulbecco's medium. In addition, higher concentrations of penicillin and streptomycin were used (100 IU/ml and 100 μg/ml, respectively). Batches of 3 ml cell suspension were mixed with 1 ml of test solution.

2.2. *Test solution*

The test solution contained 4 μM DMPHN, prepared as in [8] with tracer amounts of [^{3}H]DMPHN (own preparation), in PBS, and $^{45}Ca^{2+}$ (Amersham) as tracer. The test solution for controls contained $^{45}Ca^{2+}$ only. By addition of 1 ml of the test solution to 3 ml cell suspension containing FCS with 4.5 mM Ca^{2+} (determined by

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atomic absorption) the final $Ca^{2+}/^{45}Ca^{2+}$ concentration with 0.45 mM, with a specific activity of 1 mCi/mmol. The final toxin concentration was 1 μM.

2.3. *Assay procedure*

After 3 h culturing the medium was removed by suction. The attached cells were rinsed 3–6 times with 2 ml of NaCl (0.9%)/$CaCl_2$ (2 mM) to remove unsequestered $^{45}Ca^{2+}$ and ^{3}H-labelled toxin. Each washing step lasted for 8 s. After the last washing step the cells were dissolved in 1 ml of 0.1 N NaOH containing 0.5% SDS. One aliquot was counted in a liquid scintillation counter for ^{3}H and ^{45}Ca, while another one was used for protein determination as in [9] using bovine serum albumin as standard.

In a second series of experiments, cells incubated as described above were washed 3 times, before 2 ml of 10 μM FCCP (Fluka, Buchs) and of 10 μM A23187 (Boehringer, Mannheim), respectively, dissolved in prewarmed solutions of Häms F12 without Ca^{2+} and Mg^{2+}, were added. As a control, samples were incubated with 2 ml of Häms F12 only. After 5 min the solutions were removed, and the cells washed 3 times with NaCl (0.9%)/Ca^{2+} (2 mM), and analysed as described above.

3. RESULTS

Hepatocytes, isolated under sterile conditions and cultured for 3 h, were mainly attached to the bottom of the plastic dishes. They showed high viability (~95%) as measured by trypan blue exclusion. When incubated during the 3 h with 1 μM [^{3}H]DMPHN [8], the cells incorporated about 50 ng toxin/mg protein. This amount of toxin was high enough to cause blebbing of the cells [10]. Nevertheless the hepatocytes largely remained attached to the bottom of the dishes.

Removal of the supernatant by suction allowed several fast washing steps of the attached cells. During washing the $^{45}Ca^{2+}$ content decreased step by step. Under constant conditions, each washing step removed a constant amount (~0.09 nmol) of $^{45}Ca^{2+}$ per mg protein (fig.1a). The linear decrease allowed the extrapolation of the total amount of $^{45}Ca^{2+}$ exchanged during 3 h incubation. Assuming that the leakage of sequestered $^{45}Ca^{2+}$ during the first two washing steps was comparable to that

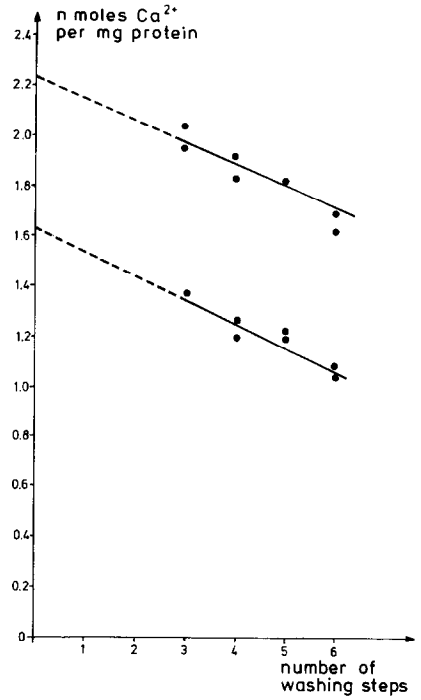

Fig.1. Decrease in the $^{45}Ca^{2+}$ exchanged during 3 h incubation of hepatocytes, by repeated washing steps in the absence (top) and presence (bottom) of 1 μM phallotoxin.

of the following steps, the total exchange of $^{45}Ca^{2+}$ was 2.24 ± 0.19 nmol per mg protein (mean ± SD, n = 42).

In [^{3}H]DMPHN-treated cells the amount of incorporated toxin remained constant during all washing steps, while $^{45}Ca^{2+}$ was also found to decrease (fig.1b). The rate of $^{45}Ca^{2+}$ loss turned out to be similar (or identical) to that in unpoisoned cells. In these experiments the extrapolation amounted to only 1.60 ± 0.15 nmol $^{45}Ca^{2+}$ per mg protein (n = 38). This value is 29% lower than in the control cells.

Cells after the third washing step containing either 2.0 or 1.4 nmol $^{45}Ca^{2+}$ per mg protein (fig.2, a and a′ respectively) were used to detect the origin of the released $^{45}Ca^{2+}$. The cells were incubated with FCCP (fig.2, c and c′) or A23187 (fig.2, d and d′). To discern the amount of $^{45}Ca^{2+}$ released unspecifically during the 5 min incubation, controls with buffer only were run in parallel (fig.2, b and b′). As fig.2 shows, the unexchangeable part of $^{45}Ca^{2+}$ (stippled areas) is nearly the same in nor-

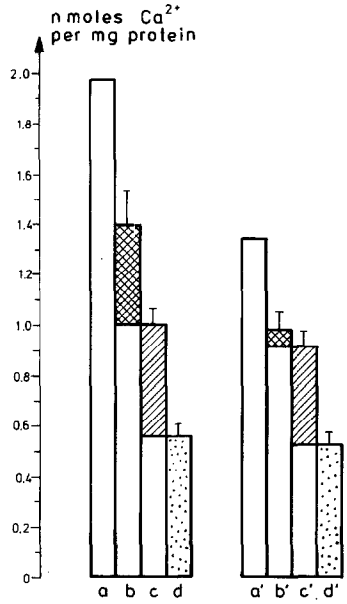

Fig.2. Decrease in sequestered $^{45}Ca^{2+}$ in hepatocytes by treatment for 5 min with buffer (b,b'), 10 μM FCCP (c,c') and 10 μM A23187 (d,d'). $^{45}Ca^{2+}$ content before treatment: a,a'. For $^{45}Ca^{2+}$ exchange the cells were cultured for 3 h in either the absence (left) or presence (right) of 1 μM phallotoxin. Stippled areas, $^{45}Ca^{2+}$ not exchanged during 5 min; hatched areas, non-mitochondrial $^{45}Ca^{2+}$ pool; double-hatched areas, mitochondrial $^{45}Ca^{2+}$ pool.

mal and phalloidin-treated cells (0.56 and 0.53 nmol $^{45}Ca^{2+}$ per mg protein). Likewise, the non-mitochondrial compartments (hatched areas in fig.2) are of comparable size, namely 0.45 and 0.38 nmol $^{45}Ca^{2+}$ per mg protein. In contrast, there was a large difference detected in the mitochondrial pools, characterized by the difference of FCCP-treated cells and buffer-incubated cells (double-hatched areas). Control cells released about 0.4 nmol $^{45}Ca^{2+}$ while the phalloidin-poisoned cells released only 0.07 nmol $^{45}Ca^{2+}$ per mg protein. This result suggests that mitochondria are the main source of the $^{45}Ca^{2+}$ released by the mushroom toxin.

4. DISCUSSION

Isolated hepatocytes, when attached to the bottom of a culture dish, can be washed quickly (8 s per step) without centrifugation or filtering procedures. Thus they provide an excellent system for studying the Ca^{2+} movements of hepatocytes. During 3 h of culturing, such cells sequester 2.2 nmol $^{45}Ca^{2+}$ per mg cell protein, an amount which is in good agreement with values of exchangeable Ca^{2+} reported by others [11,12].

For poisoning a chemically modified phallotoxin, DMPHN, was used. It has the advantage of being available in a labelled form, [^{3}H]DMPHN, allowing the measurement of the amount of toxin incorporated. No difference is known between the toxicity of DMPHN and phalloidin itself; each has an LD_{50} = 2.0 mg per kg white mouse [13]. During 3 h incubation the cells incorporated about 50 ng [^{3}H]DMPHN per mg protein. Concomitantly, the amount of $^{45}Ca^{2+}$ sequestered dropped from 2.2 to 1.6 nmol per mg protein. We conclude that the phallotoxin had irreversibly damaged a Ca^{2+}-sequestering compartment in the hepatocytes. The compartment is characterized by a capacity of about 0.6 nmol Ca^{2+} per mg protein.

In 1970, the author in [14] had investigated the lesions caused by phalloidin in a perfused rat liver, and found one of the first events to be the efflux of Ca^{2+}, occurring only 2 min after administration of the toxin to the perfusion medium. The amount of Ca^{2+} released was 220 ± 80 nmol per g liver, or, after reduction according to [6], 0.79 ± 0.29 nmol per mg parenchymal protein. Taking into account that we are comparing values for a perfused liver and for cultured hepatocytes, we feel that the value of 0.79 nmol is comparable to the value of 0.60 nmol determined here. We conclude that the same Ca^{2+} compartment was depleted in the two types of experiments.

Using FCCP and A23187 for the identification of the different Ca^{2+} compartments in hepatocytes, as described in [15], the compartment affected by the phallotoxin was clearly identified as the mitochondrial Ca^{2+} pool. In the mean of about 40 experiments this pool was found to be decreased by 81%. For comparison, the non-mitochondrial Ca^{2+} pool was decreased by about 15% and the pool of the non-exchangeable Ca^{2+} by only 6% (fig.2).

Effects of phallotoxins on mitochondria were found as early as 1956, when it was reported that 10^{-5} M phalloidin inhibits the oxidative phosphorylation in isolated mitochondria [16]. Only a few years later it was reported that

phalloidin disturbs mitochondrial functions [17] and depletes the liver of ATP [18]. However, interest in such effects declined when the highly specific interaction of phalloidin with actin was detected [19]. The strong interaction, characterized by a $K_d = 3.6 \times 10^{-8}$ M [20], suggested effective doses of phalloidin much lower than the effective dose in the experiment with mitochondria. On the other hand no causal connexion between changes in cellular actin on the one hand and the numerous phalloidin effects in hepatocytes (review [13]) on the other has ever been shown.

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